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# Sensitivity towards DMI fungicides and haplotypic diversity of their CYP51 target in the *Mycosphaerella graminicola* population of Flanders

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## Abstract

Septoria leaf blotch, caused by the fungus *Mycosphaerella graminicola*, is the most important wheat disease in Northwestern Europe, and is currently controlled by fungicide applications. Since the spread of resistance to methyl benzimidazole carbamates (MBCs) and quinone outside inhibitors (QoIs) in European countries, reliable control is mainly dependent upon sterol 14α-demethylation inhibitors (DMIs). In the last decades however, a slow shift towards reduced sensitivity of *M. graminicola* to DMIs has been observed. This shift is caused mainly by mutations in the *CYP51* gene encoding the 14α-demethylase target protein for these fungicides. In this work, *M. graminicola* isolates were sampled at fields spread over Flanders, Belgium. *In vitro* assays were used to analyze the sensitivity of the Flemish *M. graminicola* population towards different DMIs. Sequencing of the *CYP51* gene of these isolates allowed us to identify and map the haplotypes in this population. The results showed that there is a large variability in DMI sensitivity between the isolates, even within one field, which is reflected in a high diversity in *CYP51* haplotypes within the *M. graminicola* population in Flanders. Next to some haplotypes that were not described in literature before, we found that the population is dominated by *CYP51* haplotypes which were previously associated with increased resistance towards DMIs.

**Key words:** demethylation inhibitor, fungicide resistance, septoria leaf blotch

## Introduction

Septoria leaf blotch (STB), caused by the ascomycete fungus *Mycosphaerella graminicola* (anamorph: *Septoria tritici*), is one of the most important foliar wheat diseases worldwide, causing significant yield and quality losses every year. Disease control has been mainly based on chemicals, as fully resistant wheat cultivars are not available. The use of methyl benzimidazole carbamates (MBCs) and quinone outside inhibitors (QoIs) has declined because of the emergence of resistance in the field (Fraaije et al. 2007, Torriani et al. 2009). Resistance against QoIs spread relatively quickly as it is based on a single point mutation G143A in the target cytochrome *b* (Fraaije et al. 2003). Demethylation inhibitors (DMIs), of which the azole compounds constitute the largest group, are now most widely adopted to control STB.

DMIs were introduced as agricultural fungicides in the late 1960s and have become the largest and most important group of systemic fungicides in modern days. They have a site-specific mode of action and exhaustive use of the chemicals in agriculture as well as medicine, has led to the development of resistance in several organisms (Délye et al. 1997, Kunz et al. 1997, Romero & Sutton 1997; Wyand & Brown 2005). In Northwestern Europe, they have been used extensively in the control of leaf blotch in wheat since the 1980s. Unlike MBC and QoI fungicides, DMIs have not yet completely lost their efficacy, but a shift in the *M. graminicola* population towards reduced sensitivities *in vitro* and in the field has been reported in Europe since the 2000s (Mavroeidi & Shaw 2005; Clark 2006; Stammler & Semar 2011). Due to this shift, new-generation succinate dehydrogenase inhibitors (SDHIs) have been developed and are now more widely adopted in cereals, often in combination with DMIs, which still form the backbone for STB control in wheat (Fraaije et al. 2012).

DMI fungicides target the 14α-demethylase protein, a P450 enzyme playing a key role in ergosterol biosynthesis in fungi. The protein is encoded by the *CYP51* gene, consisting of three exons interrupted by two introns. Three mechanisms have been observed to account for the resistance to DMIs in *M. graminicola*: (i) enhanced efflux of the compounds by overexpression of efflux proteins, such as ABC transporters and major facilitator proteins (Stergiopoulos et al. 2003), (ii) elevation of the CYP51 level by enhanced expression of the *CYP51* gene (Stergiopoulos et al. 2003, Leroux & Walker 2011, Cools et al. 2012), and (iii) decreased affinity of the target site for the inhibitor by mutations in *CYP51*. The last mechanism is the most commonly reported and probably the most important in field resistance in Europe, at least until recent years (Leroux & Walker 2011). Several studies described the presence and accumulation of various mutations in the CYP51 target that lead to altered sensitivity to various DMIs (Cools et al. 2005, Fraaije et al. 2007, Leroux et al. 2007, Brunner et al. 2008, Stammler et al. 2008, Cools et al. 2011, Stammler & Semar 2011), which are reviewed in Cools & Fraaije (2013).

In Flanders (Belgium), the intensification of wheat production since the late 1960s was soon followed by an increase in STB, as no STB-resistant wheat cultivars were available. DMI fungicides were introduced in the mid-1970s and a

gradual decrease in their field efficacy has been observed in the last decades. As monitoring of resistance in the *M. graminicola* populations of different regions is vital for the management of STB disease, the objective of this research was to evaluate the response towards various DMIs in the population in Flanders and to map the genetic variation in the *Mycosphaerella graminicola CYP51* (*MgCYP51*) gene. Furthermore, we wanted to test whether the haplotypic variation explains variation for DMI sensitivity in the *M. graminicola* population. Hereto, the *CYP51* gene was sequenced from isolates collected in the main wheat growing areas in Flanders and sensitivity of the isolates to several commonly used DMI fungicides was assessed.

## Materials and methods

### Fungal isolates

In total, 159 isolates of *M. graminicola* were sampled from upper leaves of winter wheat during the growing season 2010 (110) and 2011 (49). Sampling was done randomly over the field at 23 locations in the main wheat growing areas of Flanders. These included conventional (9) as well as organic (5) fields and varietal field experiments (9) where the 2 most susceptible and the 2 most resistant wheat varieties were sampled. Two different time points were included: before the first (T1; GS 32-39) and after the second (T2; GS 59-65) fungicide application. Data on tillage history and pesticide use were collected for each sampling location. At variety trial sites, DMIs were applied as solo compounds or tank mixes, at GS 38 and GS 45-47. Trials were designed with four randomized replicates treated with DMIs and untreated controls. DMIs were applied at recommended field rate with a backpack sprayer using an application volume of 300 l $ha^{-1}$ and a pressure of 2,5 bar. Field rates are 125 g active ingredient (a.i.) per ha for epoxiconazole (Opus®, BASF), 90 g a.i. $ha^{-1}$ for metconazole (Caramba®, BASF), 250 g a.i. $ha^{-1}$ for tebuconazole (Horizon®, Bayer), 200 g a.i. $ha^{-1}$ for prothioconazole (Input Pro®, Bayer) and 450 g a.i. $ha^{-1}$ for prochloraz (Sportak®, BASF). Single spore isolates were obtained from cirrhi exuding from pycnidia on leaves, by plating a dilution on Czapek-Dox medium from which one colony was isolated.

### Disease assessment

Disease pressure by natural inoculum in varietal experiments was assayed at GS 65-72 by estimating the % leaf area infected of the 3 top leaves of 2 susceptible and 2 resistant cultivars, on 60 random plants per object.

### Fungicide sensitivity assays

Sensitivity of single spore isolates towards DMIs was assessed using a 96-well microtitre plate assay according to the method described by Pijls et al. (1994). DMIs tested were formulations of the triazoles metconazole, tebuconazole, epoxiconazole and prothioconazole and the imidazole prochloraz. Initially, single spore suspensions of $10^5$ and $10^7$ spores $ml^{-1}$ were tested for microtiter assays, the latter giving the best results, so this dose was used in further experiments. Spore suspensions of each single spore isolate were prepared in sterile water. Each row of a microtitre plate was filled with an isolate by adding 20 µl of the spore suspension to 160 µl PDB and 20 µl of the formulated fungicide to each well. The last row was filled with sterile water to measure the background absorbance due to fungicide and medium. Fungicide concentrations tested were field concentration based on the registered rate and 300 l water volume per ha (416 mg $l^{-1}$ epoxiconazole, 833 mg $l^{-1}$ tebuconazole, 300 mg $l^{-1}$ metconazole, 667 mg $l^{-1}$ prothioconazole and 1500 mg $l^{-1}$ prochloraz), and 1/10, 1/100, 1/300, 1/1000, 1/3000 and $1/10000^{th}$ thereof. Plates were incubated in the dark at room temperature. Fungal growth was assessed after 2 weeks by measuring the absorbance at a wavelength of 405 nm using a photospectrometer, blanks were subtracted from all columns. Per isolate, 5 replicates were measured. ED50 values for each DMI per isolate were calculated by Probit analysis (Finney 1971). Outliers (> 3xSD) were omitted from analysis.

### Molecular haplotyping

Genomic DNA was extracted from lyophilized cells of cultures of single spore isolates grown on PDB using an adaptation of the CTAB method (Doyle & Doyle 1987). DNA was suspended in TE buffer and concentrations were adjusted to 10 ng $µl^{-1}$ before storing at −20°C. Amplification of the *CYP51* gene was performed using four distinct PCR reactions, resulting in 4 overlapping amplicons. Primers for PCR isolation of the *CYP51* sequence were according to Leroux et al. (2007). PCR reaction volume was 40 µl, using 100 ng of genomic DNA, 1 µM of each primer, $MgCl_2$ 2.5 mM, dNTPs 1 mM and 0.4 µl Taq polymerase (5 U $µl^{-1}$, Thermo Scientific Molecular Biology, USA). Thermal cycling conditions were as follows: initial denaturation (5 min at 95°C), 35 cycles of denaturation (30 s at 95°C), annealing (45 s at 60°C) and primer extension (1 min at 72°C), followed by a final extension of 10 min at 72°C. Amplified fragments were subsequently sequenced on both strands by Sanger sequencing (LCG Genomics, Germany). Retrieved sequences were aligned with published *M. graminicola CYP51* cDNA sequences using the BioEdit Sequence Alignment Editor (Hall 1999), and the coding sequence was translated into an amino acid sequence for each isolate. Haplotype was determined for 110 isolates from 2010 and 30 from 2011. Haplotypes were subsequently classified into R-types according to Leroux & Walker (2011) and Stammler & Semar (2011).

### Statistical analysis

A Fisher exact test was used to test whether R-type frequencies were dependent on sampling time or location. To detect differences in distributions of ED50 values for each DMI

across R-types, sampling time point and sampling year, a Kruskal-Wallis test was used, combined with a Mann-Whitney test with a sequential Bonferroni correction for multiple comparisons. To find correlations between R-types and ED50 values, a Spearman correlation analysis was executed. All data were analyzed using SPSS software, version 19.

## Results

### Disease assessment

To get an overall idea of the disease pressure in each year, natural infection of *M. graminicola* was monitored on 2 susceptible and 2 relatively resistant wheat cultivars on 9 variety trial plots in different locations in Flanders, at sampling time point T2 at the end of the growing season. In 2010, disease pressure varied between different regions; results for 4 fields at different locations across Flanders are depicted in Fig. 1. In general, disease pressure was low in most locations, with infected leaf area being below or around 20% on the third leaf. Disease pressure was exceptionally higher in some locations in the main wheat growing area in the center of Flanders (e.g. Verrebroek), with up to 60% infected leaf area. In most locations, susceptible cultivars (Fortis, Istabraq, Mulan and Potenzial) were clearly distinguishable from more resistant ones (Homeros and Julius) (Fig. 1). In 2011, infected leaf area was below 1% in all sampling locations (data not shown).

### DMI sensitivity distribution of *M. graminicola* field isolates in Flanders

The sensitivity distributions of the *M. graminicola* population in Flanders described in this study are based on the ED50 values of individual single spore isolates derived from cirrhi from a *M. graminicola* pycnidium sampled on the upper leaves. To monitor a change in sensitivity within one growing season towards the tested azoles in the population, sampling was done at two different time points: before the first (T1; GS 32-39) and after the second (T2; GS 59-65) fungicide application. Table 1A presents the range and median of the ED50 values of the isolates per compound tested, for the two different sampling time points in 2010. Results show that the sensitivity range of isolates towards the different DMIs is large, with some high values, while the smallest range is noted for metconazole. Isolates show median ED50 values higher than 1 for tebuconazole and prothioconazole at both T1 and T2, while median ED50 values for prochloraz are lowest. Although there is a shift towards higher ED50 values at T2 for all DMIs tested, the shift was statistically significant

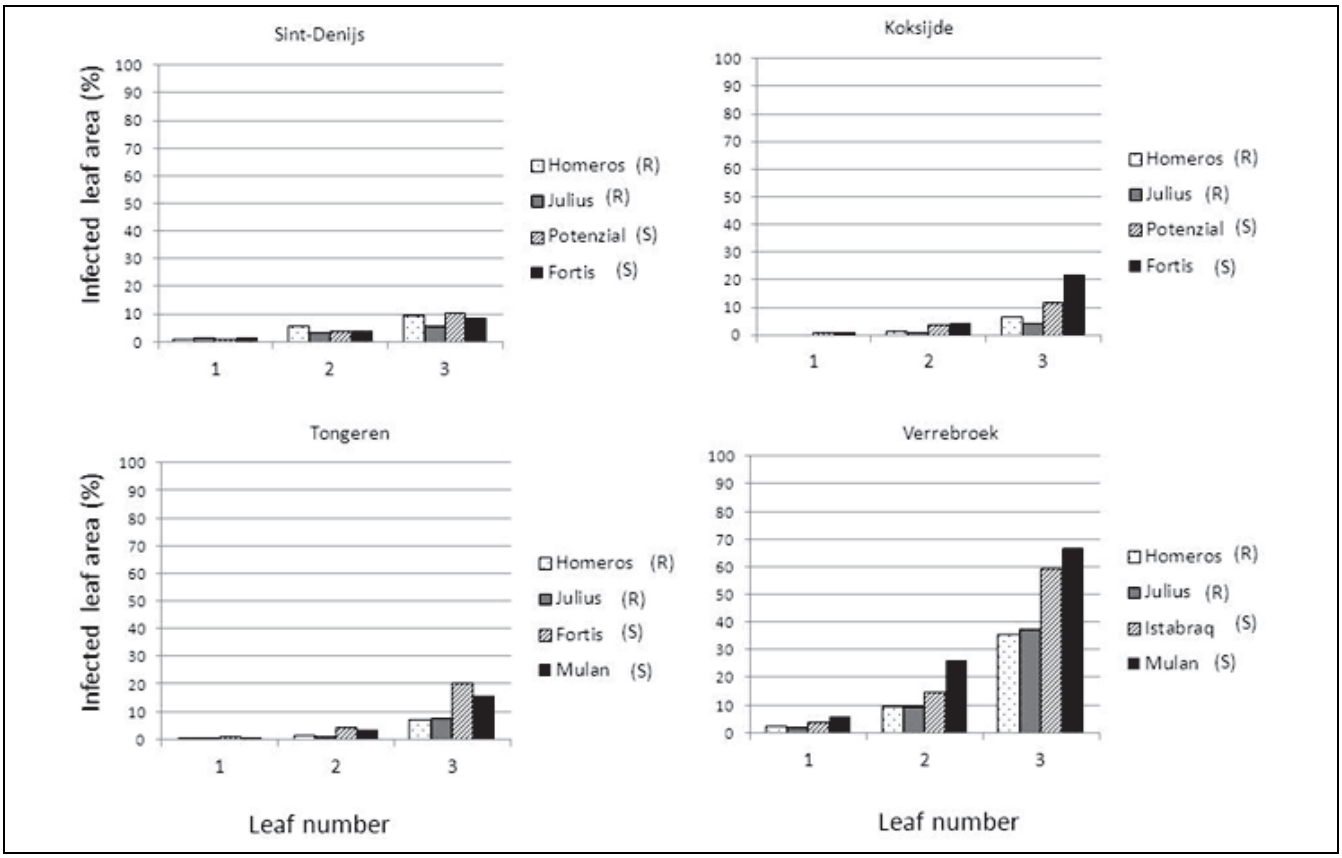


Fig. 1: Disease pressure in variety trial plots at 4 representative locations spread over Flanders. Disease pressure is expressed as percentage infected leaf area, for the three top leaves. Locations are: Sint-Denijs (south west), Koksijde (west), Tongeren (east) and Verrebroek (north). Disease pressure was assessed on two most resistant cultivars ((R), Homeros and Julius) and two most susceptible cultivars ((S), Istabraq, Fortis, Mulan or Potenzial) at each location.

Table 1: Sensitivity distribution (ED50 values in mg $l^{-1}$) of *M. graminicola* isolates to the 5 tested DMIs. n: Sample size

| | | Epoxiconazole | Tebuconazole | Metconazole | Prothioconazole | Prochloraz |
|---|---|---|---|---|---|---|
| A. Sampling year 2010 | | | | | | |
| T1 (GS 32-39) | Median | 0.15 | 1.47 | 0.16 | 1.22 | 0.026 |
| | Range | < 0.001 – 2.58 | 0.006 – 16.99 | < 0.001 – 0.61 | 0.007 – 7.47 | < 0.001 – 2.17 |
| | n = 51 | | | | | |
| T2 (GS 59-65) | Median | 0.20 | 1.78 | 0.16 | 1.22 | 0.030 |
| | Range | < 0.001 – 8.52 | < 0.001 – 25.23 | 0.032 – 1.43 | 0.058 – 11.44 | < 0.001 – 2.56 |
| | n = 42 | | | | | |
| B. Sampling year 2010, T1 + T2, location Tiegem | | | | | | |
| Median | | 0.24 | 1.44 | 0.16 | 1.57 | 0.023 |
| Range n = 23 | | < 0.001 – 8.52 | 0.14 – 21.97 | 0.087 – 0.58 | 0.007 – 8.64 | < 0.001 – 2.17 |
| C. Sampling year 2011, T1 | | | | | | |
| Median | | 0.66 | 5.9 | 0.33 | 1.41 | 0.22 |
| Range n = 49 | | 0.028 – 9.79 | 0.023 – 21.38 | 0.015 – 1.55 | 0.002 – 9.31 | < 0.001 – 2.38 |

only for epoxiconazole ($P < 0.05$). Distributions of ED50 values for all tested isolates at one location (Tiegem) are presented in Table 1B. These data show that even within one field, a wide variability in ED50 values is observed, with a similar variation and median as observed over all the sampling locations.

In 2011, disease pressure was too low to allow sampling at T2. Table 1C presents the distributions of the ED50 values of isolates sampled at T1 (GS 32-39). As for isolates from 2010, median ED50 values are highest for tebuconazole and prothioconazole, but ED50 values for all DMIs except prothioconazole are significantly higher compared to those of isolates sampled in 2010. The largest shift was found for prochloraz, with the median ED50 value being 10 times higher.

There was no significant overall correlation of sensitivities of the isolates to the different DMIs ($P \geq 0.05$). Due to the very low disease incidence in 2011, the size of our data set did not allow to find a correlation between disease pressure at one location in 2010 and ED50 value range or median in 2011. Moreover, no correlation between distribution of sensitivities and cultivar, cropping history or prior fungicide use was found.

**CYP51 haplotype and R-type diversity in Flanders**

Haplotyping of the isolates was done by sequencing of the *CYP51* coding region. Wild-type haplotypes were not found in the population in Flanders. Of the mutations with a known effect on resistance to DMIs, variations at amino acid residues 136, 137, 379, 381, 459–461 and 524 have been reported to be most important. In the Flemish population, deletions or mutations in the region 459–461 are most predominantly present (in 95% of the isolates). The amino acid change I381V, conferring resistance to tebuconazole, was present in 81% of the isolates. Y137F was not found in any of the isolates. V136C was more commonly observed than V136A in the Flemish *M. graminicola* population, in 17% vs. 7% respectively. S524T was found in 2 isolates only, in combination with D107V, I381V and N513K.

Haplotypes were classified into R-types according to Leroux et al. (2007), Leroux & Walker (2011) and Stammler & Semar (2011). Fig. 2 shows the frequencies of the haplotypes observed in the Flemish population in the growing seasons 2010 and 2011. R6 and R8 are clearly the most prominent

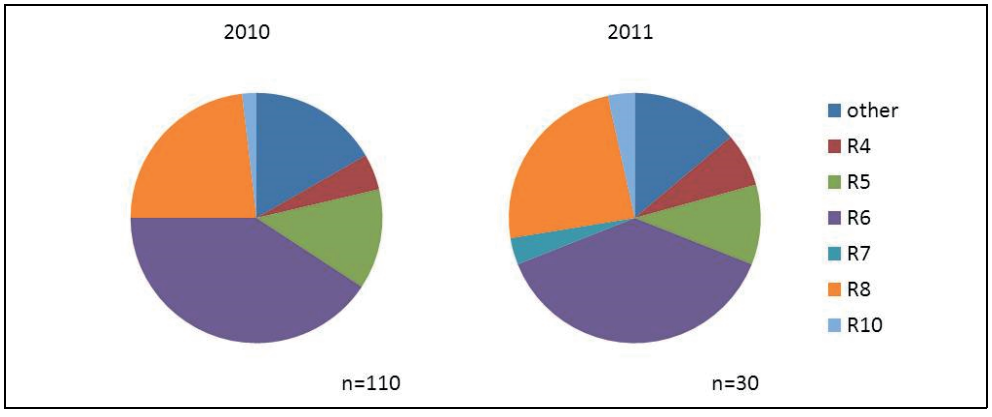


Fig. 2: Distribution of R-types in the *M. graminicola* population in Flanders in 2010 and 2011. Classification of the various *CYP51* haplotypes into R-types is based on Leroux & Walker 2011 and Stammler & Semar 2011. n: Sample size.

R-types in both years, found respectively in 42% and 23% of the population in 2010 and in 38% and 24% in 2011. No R-types below R4 were found in any of the field isolates. We also detected isolates which could not be classified according to this system and were classified as 'other' (15 in 2010 and 4 in 2011). Haplotypes within this class were previously reported [D107V + I381V + N513K + S524T], and combinations unreported so far: [L50S + V136C + S188N + I381V + del 459/460], [L50S + V136C + S188N + I381V + del 459/460 + N513K] and [L50S + V136C + S188N + A379G + I381V + del 459/460 + N513K]. There was no statistically significant difference in R-type frequencies between the two sampling time points T1 and T2 in 2010, nor between the two years (data not shown).

Table 2 depicts distributions of the ED50 values determined for isolates of 2010, within the different R-types, per DMI tested. ED50 values display a broad range of sensitivities within each of the R-types, with ED50 values of the different R-types largely overlapping. Based on a Kruskal-Wallis test followed by Bonferroni correction, the distribution of ED50 values for each DMI is not significantly different across all R-types. There was no significant correlation between ED50 values and R-type ($P \geq 0.05$). However, some clear trends can be distinguished. ED50 values for prochloraz and metconazole are lowest and also vary less across R-types, especially for metconazole. Highest ED50 values for each DMI are found in the R6 class, which contains the largest number of isolates. Highest median ED50 values for tebuconazole and metconazole are found in the 'other' R-type class, while highest median ED50 values for epoxiconazole, prothioconazole and prochloraz are found in the R4 class. Similarly to the variability in ED50 values, a large variability in R-types was observed within each location (data not shown).

## Discussion

The aim of this study was to analyze the *M. graminicola* population in Flanders for its sensitivity towards different DMIs and the frequency and distribution of *CYP51* haplotypes, and their potential link.

Disease pressure was very moderate in Flanders in 2010, and general dry weather conditions were not conducive for infection and disease dispersal to the upper leaves in most regions. In the main wheat growing area in the center of Flanders, infection was higher. This could be due to locally higher precipitation. Alternatively, this could indicate the prevalence of a more pathogenic and/or more DMI-resistant *M. graminicola* population. However, our data showed no correlation between distribution of sensitivity towards the different DMIs and location. Furthermore, the infection pressure is clearly influenced by the wheat variety used. Our data confirm that climate and wheat variety greatly influence the disease pressure (Cowger & Mundt 2002, Pietravalle 2003). In 2011, disease pressure was very low, with infected leaf area below 1% in all locations, due to very dry weather conditions during the growing season.

*In vitro* sensitivity of *M. graminicola* field isolates to DMIs was assessed using a microtitre plate assay. The sensitivity towards different triazoles by determination of the ED50 values of the isolates showed a large variability over all DMIs tested, while there were no significant correlations between sensitivities for the different DMIs within isolates. Moreover, our data show that even within one field, there is a wide variability, indicating that the population in a single field can be very heterogeneous in its sensitivity towards DMIs. This is in accordance with previous findings, where extensive monitoring of European populations revealed a broad range

Table 2: Sensitivity distribution (ED50 values in mg $l^{-1}$) of *M. graminicola* isolates to the 5 tested DMIs per R-type. n: Sample size

| R-type | | Epoxiconazole | Tebuconazole | Metconazole | Prothioconazole | Prochloraz |
|---|---|---|---|---|---|---|
| R4 (n = 6) | Median | 0.57 | 0.53 | 0.16 | 2.24 | 0.29 |
| | Range | 0.16 – 1.57 | 0.078 – 1.44 | 0.085 – 0.29 | 0.32 – 3.65 | 0.003 – 2.17 |
| R5 (n = 12) | Median | 0.21 | 0.84 | 0.09 | 1.65 | 0.076 |
| | Range | < 0.001 – 0.71 | 0.13 – 2.51 | 0.015 – 1.16 | 0.22 – 4.22 | < 0.001 – 0.99 |
| R6 (n = 38) | Median | 0.14 | 1.78 | 0.17 | 0.92 | 0.024 |
| | Range | < 0.001 – 8.52 | < 0.001 – 25.23 | < 0.001 – 1.43 | 0.007 – 11.44 | < 0.001 – 2.56 |
| R8 (n = 21) | Median | 0.13 | 1.58 | 0.14 | 1.66 | 0.033 |
| | Range | < 0.001 – 1.62 | 0.029 – 14.25 | 0.043 – 0.62 | 0.023 – 5.37 | < 0.001 – 0.79 |
| R10 (n = 1) | | 0.002 | 0.62 | 0.15 | 0.68 | < 0.001 |
| Other (n = 15) | Median | 0.11 | 2.41 | 0.19 | 1.44 | 0.007 |
| | Range | < 0.001 – 2.50 | 0.55 – 24.30 | 0.045 – 0.34 | 0.52 – 6.71 | < 0.001 – 1.05 |

of ED50 values within one trial location (Mavroeidi & Shaw 2005, Stammler & Semar 2011). The sensitivity distributions in the Flemish population show a moderate to high level of resistance when compared with the lowest values noted for each DMI, conform to reports from other European countries (Mavroeidi & Shaw 2005, Leroux et al. 2007, Stammler & Semar 2011). Median ED50 values for prothioconazole and tebuconazole are higher than for other tested DMIs and lowest for prochloraz, similar to findings reported for France (Leroux et al. 2007). Epoxiconazole, prothioconazole, tebuconazole and metconazole have been used extensively to control STB in Flanders, while prochloraz has not been used lately, which may be reflected by a persistent high *in vitro* efficacy for this compound.

Sampling before and after fungicide application allowed us to analyze selection pressure by fungicide application within one growing season. We did observe a small but consistent shift for most DMIs towards higher ED50 values at T2 compared to T1, which was statistically significant for epoxiconazole. This indicates that during the growing season 2010, a moderate shift towards more resistant isolates occurred. Isolates from 2011 showed significantly higher ED50 values compared to 2010, for all tested DMIs except for prothioconazole. The median ED50 value of prochloraz was found to be 10 times higher in 2011. Prochloraz is a non-systemic fungicide with a short action-period, not favouring selection. Together with the fact that prochloraz is not extensively used in the field, this result suggests co-selection towards lower sensitivity by other DMIs. In Flanders, epoxiconazole is mostly used to control STB, followed by prothioconazole and tebuconazole. Cases of positive as well as negative cross-resistance between several triazoles and prochloraz have been reported for *M. graminicola* and for several other pathogens (Leroux et al. 2000, 2007). In 2007, Fraaije et al. found that substitution I381V was selected for by tebuconazole and difenoconazole treatment, while prochloraz treatments selected negatively for I381V. These findings implicated that treatment with different DMIs could offer a strategy for resistance management. However, the authors pointed out that reduced sensitivity does not depend on one mutation only, and additional resistance mechanisms are usually present in the population. Our results corroborate this and further demonstrate that positive co-selection can occur between azoles and prochloraz. For prothioconazole, we found no shift towards higher ED50 values in 2011, which might point to a lower selection pressure for this compound. This could explain why it is still effective to control STB in the field, although it has been used extensively since its introduction in 2001. The higher efficacy of this compound can possibly be linked to its different mode of binding CYP51 compared to other azoles (Mullins et al. 2011). Additionally, epoxiconazole and metconazole have good field efficacy till date, which is reflected in a lower selection pressure in the field for these compounds in our dataset, than for prochloraz and tebuconazole. However, it should also be noted that other factors than *in vitro* efficacy influence the eventual field efficacy of a compound, and the link between *in vitro* and field efficacy is not always straightforward (Stammler et al. 2008).

Our data show that quite large shifts in sensitivities can occur over the seasons, and more limited shifts during one growing season. This is in accordance with the fact that during the growing season, growth is dominated by production of clonal pycnidiospores, which are dispersed by rain splash over distances of a few meters, and sexual reproduction takes place to a lesser extent (Zhan et al. 1998, 2007). New infection cycles usually involve air-borne ascospores which can be transferred over long distances. One study similarly reported weak selection for flutriafol resistance to occur in field experiments over a single season (Pijls & Shaw 1997). Fraaije et al. (2005) reported a drastic increase in resistance towards a QoI fungicide in an airborne *M. graminicola* ascospore population after one application and within the growing season, correlated with an increase in R-allele frequency from 35 to 80%. Resistance in this case is however based on one mutation in cytochrome *b* (G143A), in contrast to resistance to DMIs, which might explain why we didn't observe such a drastic shift within one season. Changes in sensitivity to DMIs over the years have been reported to have occurred since the 1980s, and to be accelerating over the past few years (Mavroeidi & Shaw 2005, Leroux et al. 2007, Cools et al. 2011). This phenomenon of acceleration might be linked to the increasing frequency of mutations in the *CYP51* gene in the population. However, our dataset comprising two growing years only, cannot rule out natural cycles in sensitivity that might occur over years. Correlations between ED50 values and wheat cultivar, cropping and tillage history, and fungicide application were not detectable, as the number of isolates was too limited for this kind of correlation studies due to low disease pressure during the sampled years. Especially in 2011, weather conditions were unconducive to infection, indicating that probably more highly virulent strains were collected. Together with their ED50 values being significantly higher than the year before, this confirms a link between virulence and fungicide resistance which has been observed in some recent studies (Zhan et al. 2007, Yang et al. 2013).

*CYP51* sequence analysis of the isolates allowed us to study the haplotype structure and distribution within the Flemish population. Wild-type haplotypes were not found at any of the locations. We also did not detect Y137F, one of the oldest CYP51 mutations dominating the Western European population during the 1990s, but now very rare or even absent (Stammler et al. 2008). In general, deletions or mutations in the region 459–461 are dominating, together with I381V. The I381V mutation has been found since 2000 and has been accumulating in the European population, being a very abundant mutation these days (Cools et al. 2011, Stammler & Semar 2011). Substitutions at position 136 were also found, V136C being more commonly observed (almost 2.5 times more) than V136A in the Flemish population. Isolates carrying V136A/C have been reported to be less sensitive to prochloraz, while I381V lowers sensitivity towards most triazoles, in particular to tebuconazole (Fraaije et al. 2007, Leroux et al. 2007). We also found isolates carrying both mutations, which had not been found until quite recently (Stammler et al. 2008).

*CYP51* haplotypes were classified in R-types by Leroux et al. (2007), Leroux & Walker (2011) who described several

*in vitro* sensitivity patterns towards different DMIs for the different haplotypes, and that take into account the major amino acid changes which have been shown to affect DMI sensitivity. The classification system was later adopted and simplified by Stammler et al. (2008), Stammler & Semar (2011). When we classified the detected haplotypes into R-types, we found that R6 and R8 are the most predominant R-types in the Flemish population, which is in accordance with previous findings (Stammler & Semar 2011). The third largest group consisted of haplotypes which could not be classified according to the above-mentioned system. The haplotype [D107V + I381V + N513K + S524T] has been isolated and described before (Stammler et al. 2008). However, we also found haplotypes accumulating several mutations that were not reported in literature before: [L50S + V136C + S188N + I381V + del 459/460], [L50S + V136C + S188N + I381V + del 459/460 + N513K] and [L50S + V136C + S188N + A379G + I381V + del 459/460 + N513K].

Together with the sensitivity data, our haplotype data indicate that genotypes with reduced sensitivity to DMIs are dominating the *M. graminicola* population in Flanders, as observed elsewhere in Western Europe. Of the detected R-types, most are found even at one location, indicating large genetic variability in the field. It has been observed that *M. graminicola* populations on leaves and larger scales are genetically mixed, with the majority of regional variation present in any individual field (McDonald et al. 1999, Linde et al. 2002, Zhan et al. 2002, 2003, Mavroeidi & Shaw 2005).

When expressed in yeast, the impact of certain mutations and limited combinations thereof could be functionally determined (Cools et al. 2011). However, although haplotypes accumulating more substitutions seem to confer higher resistance, no significant correlations between R-types and sensitivity distributions could be distinguished in this study. One reason might be the complex stacking of more mutations. This confirms previous findings that the mutations might have a rather limited influence on the sensitivity towards DMI compounds, and are probably part of a multigenic resistance mechanism (Stammler et al. 2008, Leroux & Walker 2011, Stammler & Semar 2011). Surprisingly, R4 has the highest median ED50 values for epoxiconazole, prothioconazole and prochloraz. However, highest ED50 values for each of the DMIs are more often found in the higher R-classes or the 'other' class which generally accumulate more substitutions than other R-types. We can hypothesize that the haplotype determines the basal level of resistance, while additional factors and/or genes are involved in the actual resistance phenotype.

This was the first monitoring study of this scale to analyze *CYP51* haplotype diversity and DMI sensitivity in the *M. graminicola* population in Flanders. Our data show that the Flemish *M. graminicola* population shows large variability in *CYP51* haplotypes and in the sensitivities towards different DMIs, together with a high level of *in vitro* resistance towards these compounds. We also detected some *CYP51* haplotypes that, to our knowledge, were not reported in literature before. There was no correlation between *CYP51* haplotypes and sensitivities to the DMIs tested in this study, pointing again to the high heterogeneity at genetic level as well as in DMI sensitivity in this fungus and the complexity of its resistance mechanism towards DMIs.

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